RESEARCH PAPER

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Knockout of AtMKK1 enhances salt tolerance and modifies metabolic activities in *Arabidopsis*

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Mitogen-activated protein kinase (MAPK) pathways represent a crucial regulatory mechanism in plant development. The ability to activate and inactivate MAPK pathways rapidly in response to changing conditions helps plants to adapt to a changing environment. AtMKK1 is a stress response kinase that is capable of activating the MAPK proteins AtMPK3, AtMPK4 and AtMPK6. To elucidate its mode of action further, several tests were undertaken to examine the response of AtMKK1 to salt stress using a knockout (KO) mutant of AtMKK1. We found that AtMKK1 mutant plants tolerated elevated levels of salt during both germination and adulthood. Proteomic analysis indicated that the level of the α subunit of mitochrondrial H^+-ATPase, mitochrondial NADH dehydrogenase and mitochrondrial formate dehydrogenase was enhanced in AtMKK1 knockout mutants upon high salinity stress. The level of formate dehydrogenase was further confirmed by immunoblotting and enzyme assay. The possible involvement of these enzymes in salt tolerance is discussed.

Introduction

MAPK (mitogen-activated protein kinase) pathways play an important role in regulating plant growth and development.[1,2] These pathways consist of at least three core enzymes: a MAPK (MPK), activated by a MAPK kinase (MAPKK, MKK), which is in turn activated by a MAPK kinase kinase (MAPKKK, MEKK) through phosphorylation. MAPKKs/MKKs all have a common activation motif, S/TXXXXXS/T, as well as a high specificity for the downstream MAPKs.[3] In *Arabidopsis thaliana* there are 20 MAPKs, 10 MAPKKs and 60 MAPKKKs.[4]

Some fully identified pathways such as AtMEKK1-AtMKK1/AtMKK2-AtMPK4 respond to a variety of stresses including pathogen attack, wounding, salt or cold temperature.[5-9] AtMEKK1-AtMKK4/AtMKK5-AtMPK3/AtMPK6 is another identified stress pathway.[10] The number of activities that MAP kinases have been shown to impact is significant but there are still more than 60 MAPK cascade genes with unknown functions.[1] This means that the number of potential interactors for all levels of a MAPK cascade is immense. Most MAPKKs are responsible for activating a very few members of the MAPK family, but there do appear to be exceptions leading to cases of redundant activations, as seen in AtMKK1/AtMKK2 activating AtMPK4, AtMKK4/AtMKK5 activating AtMPK3/AtMPK6 and AtMKK1/AtMKK2 also being able to activate AtMPK6.[1,9,11]

A complete pathway for AtMKK1 has been identified. AtMEKK1 was shown to phosphorylate and activate AtMKK1 in a yeast two-hybrid system[5] and AtMPK4 was shown to be connected to AtMKK1 during the same yeast two-hybrid experiment.[12] Since the initial identification of the AtMEKK1-AtMKK1-AtMPK4 pathway a multitude of other interactors have been identified. AtMKK1 plays a role in the regulation of not only AtMPK4, but also AtMPK3 and AtMPK6.[6,11] The most commonly identified pathways involving AtMKK1 are wounding, bacterial pathogen response, cold, drought, salt stress, oxidative stress, touch and abscisic acid (ABA).[3,5-7,9,11] Some of the stresses such as wounding have been accepted with few conflicting data, while others, such as salt, have met with mixed responses. Although there are studies linking AtMKK1 with the activation of AtMPK4 in salt stress,[3] others refute this claim and state that AtMKK1 is not involved in the salt response.[9]

In our work to test the involvement of AtMKK1 in the salt stress response, a comparative approach using wild-type and AtMKK1 T-DNA insertion plants was followed. Knockout mutants were grown with wild-type plants and then exposed to high salinity stress. Phenotypic changes and variation in germination were measured. At the biochemical level, a proteomic analysis based on 2D-PAGE and mass spectrometry was also used.

Results

Salt stress and seed germination. T-DNA insertion was found in the third intron of AtMKK1 in *Arabidopsis* genome.[13] The

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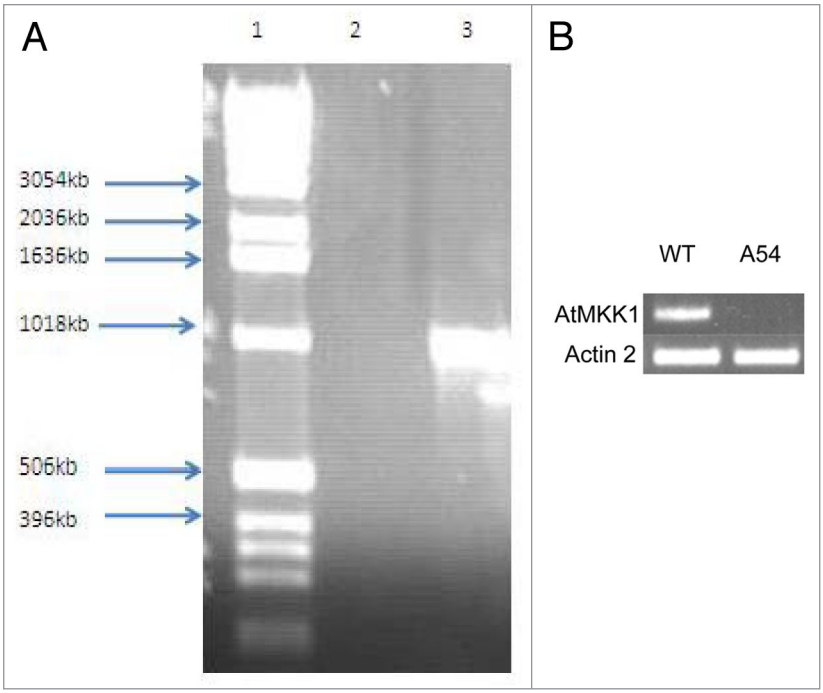

Figure 1. (**A**) Homozygous mutants for SALK_140054. Lane 1 shows the 1 kb ladder. Lane 3 shows the presence of the T-DNA insertion obtained after PCR. Lane 2 with no band shows that this sample contains the T-DNA insertion preventing PCR from running due to the size of the insert. PCR was performed twice to confirm the results. (**B**) PCR analysis of mRNA accumulation of AtMKK1 in wild-type and A54 KO plants. The upper panel shows the AtMKK1 transcript. The lower panel shows the expression of the internal control gene actin 2.

homozygous knockout status (A54 KO line) was verified (**Fig. 1A**) and the lack of AtMKK1 transcript in A54 SALK lines was confirmed by PCR (**Fig. 1B**). **Figure 2** shows the germination response of the seed lines to varying levels of salinity. Almost 100% of the seeds placed on the control plate germinated, whereas fewer than 5% of the seeds germinated on the plate amended with 250 mM NaCl in either wild-type or A54 KO plants. However, the germination rate under 125 mM NaCl condition was much higher in the A54 KO line than in wild-type plants (**Fig. 2**).

Effect of salt stress on adult plants. The second stage of salt stress testing was generated by exposing adult plants to salt stress. All of the plants used in this experiment were between three and four weeks of age. As shown in **Figure 3A** there are visible differences between the treated plants and the control plants at day 12, and the knockout mutants had a higher tolerance to salt than the wild-type plants. There were very few yellow leaves at this time point on most of the plants. When plants were subjected to salinity stress, leaf electrolyte leakage, a reflection of membrane damage, was considerably reduced in the knockout mutants (**Fig. 3B**).

Mass spectrometry identification of protein changes. **Figure 4** shows the results of two dimensional gels contrasting the proteomic profiles respectively of A54 and wild-type with both salt treated and untreated plants. **Table 1** shows the regulated proteins as determined by mass spectrometry and Mascot. The levels of the α subunit of mitochrondrial H^+-ATPase, mitochrondial NADH dehydrogenase and mitochrondrial formate dehydrogenase were lower in the KO line than in the wild-type plants, but they were upregulated when KO plants were treated with NaCl, even though NaCl treatment did not bring the levels up to those in the wild-type plants (**Fig. 4C and D**). Other proteins responded to salt in wild-type plants. Within the limited number of spots analyzed by mass spectrometry, ATG2 and a S5 ribosomal protein were downregulated when wild-type plants were treated with NaCl (**Fig. 4A and B**; **Table S1**). While the scope of this study is limited, data for all these identified spots were obtained from three biological repeats with similar results and the most noticeable up and downregulated protein profiles were the same.

Downregulation of formate dehydrogenase in AtMKK1 knockout plants. A GST-AtFDH polyclonal antibody was used to analyze the changes of protein levels of FDH further. AtFDH expression did not change with salt treatment in wild-type plants (**Fig. 5A**). In A54 KO line, the level was lower than that in the wild-type plants. This level was moderately enhanced with salt treatment (**Fig. 5A**). Enzyme assay further supported the western blotting results (**Fig. 5B**). Both analyses have confirmed the observation in 2D gels (**Fig. 4C and D**).

AtMKK1 co-expression analysis. Bioinformatic tools were used to analyze the network of genes and proteins that are associated with AtMKK1. Seven proteins were identified that are likely to interact directly with AtMKK1 (**Table 2**; **Fig. S1**). Microarray data analysis for wild-type *Arabidopsis* has indicated that 24 h treatment with 250 mM NaCl enhanced NAD-dependent FDH transcription but suppressed AtMKK1 transcription, suggesting that expression of AtMKK1 and AtFDH in response to salt stress is negatively correlated (**Fig. 6**).

Discussion

Salt is a significant source of stress in plant development affecting all stages of growth and development from germination through to seed development.[14] AtMKK1 is involved in *Arabidopsis* stress response pathways, however, the literature has presented complicated or even contradictory roles for AtMKK1.[1,9,11,15] In our present work, we studied salt tolerance in an AtMKK1 knockout line. Initially the response of knockout mutant seeds was examined by following the changes in the germination patterns of wild-type vs. knockout plants. While both wild-type and A54 KO seeds were not capable of growing when exposed to 250 mM NaCl, A54 KO seeds grew well under 125 mM NaCl. They had a much higher germination rate at this level of salt and there was a large number of plants that exhibited tolerance to the salt contained in the plates. Germination is a complicated

process regulated by genetic, hormonal and environmental factors.[16-18] Salt tolerance is a multigenic trait and the overall trait is determined by a number of sub-traits, any of which might, in turn, be determined by any number of genes.[19] These sub-traits generally include an ability to minimize the net accumulation of Na^+ and/or Cl^- ions and to select K^+ from a background of high Na^+ concentration.[19] In the context of MAPK pathways, the influence that AtMKK1 has on germination could be a direct result of its impact on its downstream targets such as AtMPK4 or AtMPK6 and their subsequent influence on transcription factors.[1] The elevated response of AtMKK1 knockout plants may also occur as a result of modification to hormone sensitivity or production, particularly ABA.[20-22] Altered responses to ABA can lead to seeds capable of breaking dormancy.[11,16,23] The incidence of germination could increase in AtMKK1 knockout plants in the presence of ABA, unlike overexpressed AtMKK1 or wild-type.[11] The AtMKK1 to AtMPK6 pathway involved in ABA signaling may permit germination in the presence of salt due to the ability of the knockout mutants to resist the dormancy control of ABA.[11] gibberellic acid (GA) is required for plants to enter germination and high salinity can negatively affect the production of GA.[16,24] The ability of AtMKK1 knockout plants to continue to grow despite the high salinity may indicate that there is a change in GA production, or that the reduced sensitivity toward basal ABA levels may allow for the germination of seedlings despite the negative pressure of high salinity on GA production. The cross talk and wide ranging interactions of MAPK pathways mean that the isolation of a single point of effect from the removal or overexpression of a gene is extremely difficult. Changing genes affecting dormancy in response to hormonal changes tend to cause plants to become insensitive or less sensitive to the effects of hormones. Examples of insensitive genes, such as abscisic acid insensitive (ABI) genes, or gibberallic acid insensitive (GAI) genes, illustrate the response of typical germination related genes.[25]

There are few examples of a role for MAPK in germination, particularly stress responsive examples such as AtMKK1. However there are multiple transcription factors such as ABI5, a bZIP family transcription factor, or ABI3, a B3 family transcription factor that both play a role in ABA signaling and therefore seed dormancy.[25,26] The downstream MAPKs from AtMKK1 have not all been identified and the further downstream transcription factors have not been identified either. The role of AtMKK1 in the activation of AtMPK4 has been well established,[1] but other targets have remained unclear. The identification is further complicated by AtMKK1 activating different proteins based on in vivo or in vitro condition.[9] Transient expression systems such as protoplasts, often provide different results when compared with whole plant studies.[9] AtMKK1 may also act on different downstream proteins based on the induction of various stress responses.

The appearance and development of the knockout mutants post-NaCl treatment showed the knockouts to be healthier and more capable of withstanding the effects of the NaCl (**Fig. 3A**). The electrolyte leakage was much lower in AtMKK1 knockout plants (**Fig. 3B**). The end result was the same, death, for both

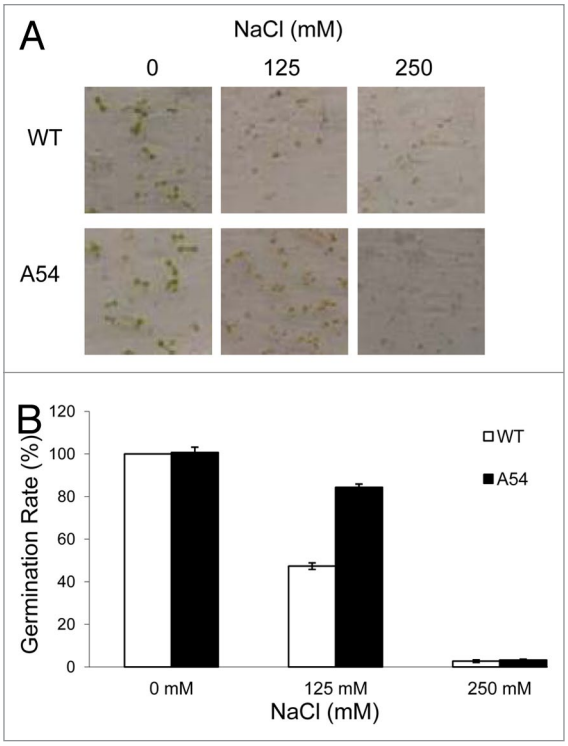

Figure 2. Seed germination under salt stress. A high salinity stress (125 mM or 250 mM NaCl) was applied to germinating seeds. After 4 d at 4°C the seeds were plated on the experimental plates and then placed in the growth chamber. Seed germination was examined 7 d after. (**A**) Seed germination on agar plates. (**B**) Germination percentage. Values are the mean ± standard deviation from three independent experiments (n = 3).

the mutants and the wild-type but the process was delayed in the AtMKK1 knockout mutants (data not shown). The role of AtMKK1 in salt stress has been debated. AtMKK1 has been shown to be activated by salt stress through increased phosphorylation post-NaCl treatment, with no indication of increased AtMKK1 gene expression, indicating that AtMKK1 was activated by an upstream kinase, likely AtMEKK1.[3,9] Our study of AtMKK1 knockout plants however shows that AtMKK1 has a place in the salt stress pathway, but likely as a negative regulator of salt stress.

Two dimensional electrophoresis identified several proteins responding to salt stress between A54 plants and the wild-type (**Fig. 4**). These were chosen for LC-MS analysis (**Table 1**). All three of these upregulated proteins appear to play a role in the production of cellular energy. ATP is a crucial component in the functioning of the affected cells in response to salt stress, providing energy to compartmentalize salt ions, or to drive ion transporters to remove salt from the cells.[27,28] The need to maintain homeostasis is crucial for plants to survive: high NaCl concentrations can reduce biomass, reproductive ability, photosynthetic activity, or if severe enough, can cause mortality.[29] The trend toward the increased expression of proteins that play a role in the production of energy is an interesting but not unanticipated one as seen in microarray data or protein-protein interaction data obtained after salt stress or biotic stress experiments in other

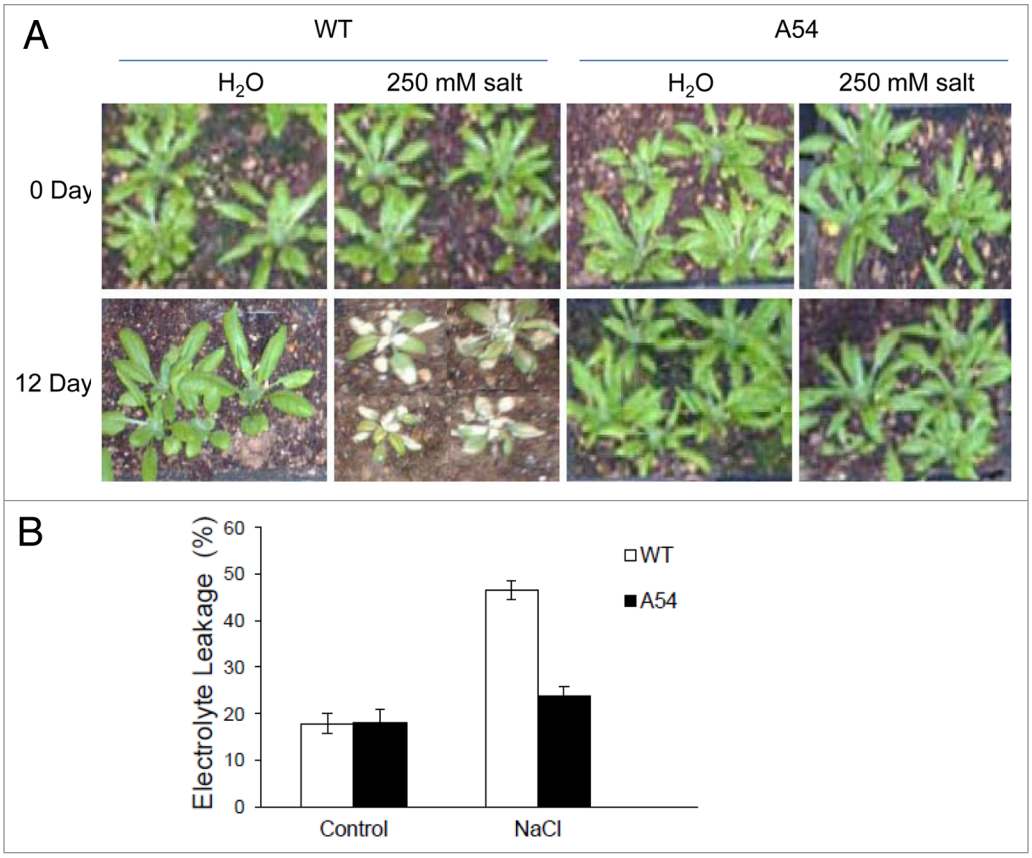

Figure 3. (**A**) Effects of high salinity on the growth of wild-type and A54 KO plants. This experiment was performed three times with similar results. (**B**) Electrolyte leakage of wild-type and A54 KO plants. Plants were grown 3 d in the presence or absence of 100 mM NaCl. Plants without treatment of NaCl were the controls. Values are the mean ± standard deviation from three independent experiments (n = 3).

species,[30,31] where energy related transcripts and proteins are consistently upregulated. Mitochondrial H^+-ATPase α subunit appeared unchanged with salt treatment in wild-type plants. In A54, the level of this protein was lower than that in wild-type plants, but increased with salt treatment (**Fig. 4**). The ability to produce energy, to control pH and to distribute ions is crucial to the ability of a plant to react to a saline environment. Recently, expression of a vacuolar ATPase subunit c1 (SaVHAc1) from a halophyte grass Spartina alterniflora was found to enhance salt tolerance of rice plants which was achieved mainly through adjustments in early stage and preparatory physiological responses.[32] In addition to its own increased expression in rice, SaVHAc1 expression also led to increased transcription of other rice genes, especially those involved in cation transport and ABA signaling.[32] H^+-ATPases can be found in the membranes of most cellular components, from vacuoles, mitochondria and chloroplasts, to the plasma membrane, indicating that the general function of this protein is crucial for the maintenance of homeostasis and the ability for a plant to survive negative pressure.[33]

The second upregulated protein was an NADH dehydrogenase. The level of this protein was below the detection limit of Coomassie Blue in the wild-type plants with or without salt treatment, or in A54 knockout line with no salt treatment, but its level in A54 increased with salt treatment. The increased production of a NADH dehydrogenase is an important step in energy production as NADH dehydrogenase is a component of the electron transport chain often providing electrons to coenzyme q.[34] The ability to accelerate the production of energy, step up photorespiration, regulate redox or even minimize reactive oxygen species (ROS) production allows the plant cell to react to changes in stress conditions such as high salinity.[35]

The third protein identified was a formate dehydrogenase (FDH). Formate dehydrogenase appeared unchanged with salt treatment in wild-type plants. In A54 knockout line, the level of this protein was lower than that in wild-type plants, but upregulated again with salt treatment even though the salt treatment did not bring it to the level observed the wild-type plant (**Fig. 4**). This observation in 2D gels was confirmed by western blotting analysis (**Fig. 5**). FDH is involved in forming single carbon units for photosynthesis and also in breaking down byproducts of glycolysis.[36] Methanol, formaldehyde and formate, all being one-carbon metabolites, appeared to induce FDH in *Arabidopsis*.[37] FDH is not always active but FDH transcript accumulation has been reported under various abiotic and biotic stresses including hypoxia, chilling, drought, dark, wounding and pathogen attack in potato, common bean and *Arabidopsis*,[36,38,39] as well as iron deficiency and anaerobia in barley root.[40] It is not a large leap to the possibility that FDH can play a role in the stress response to

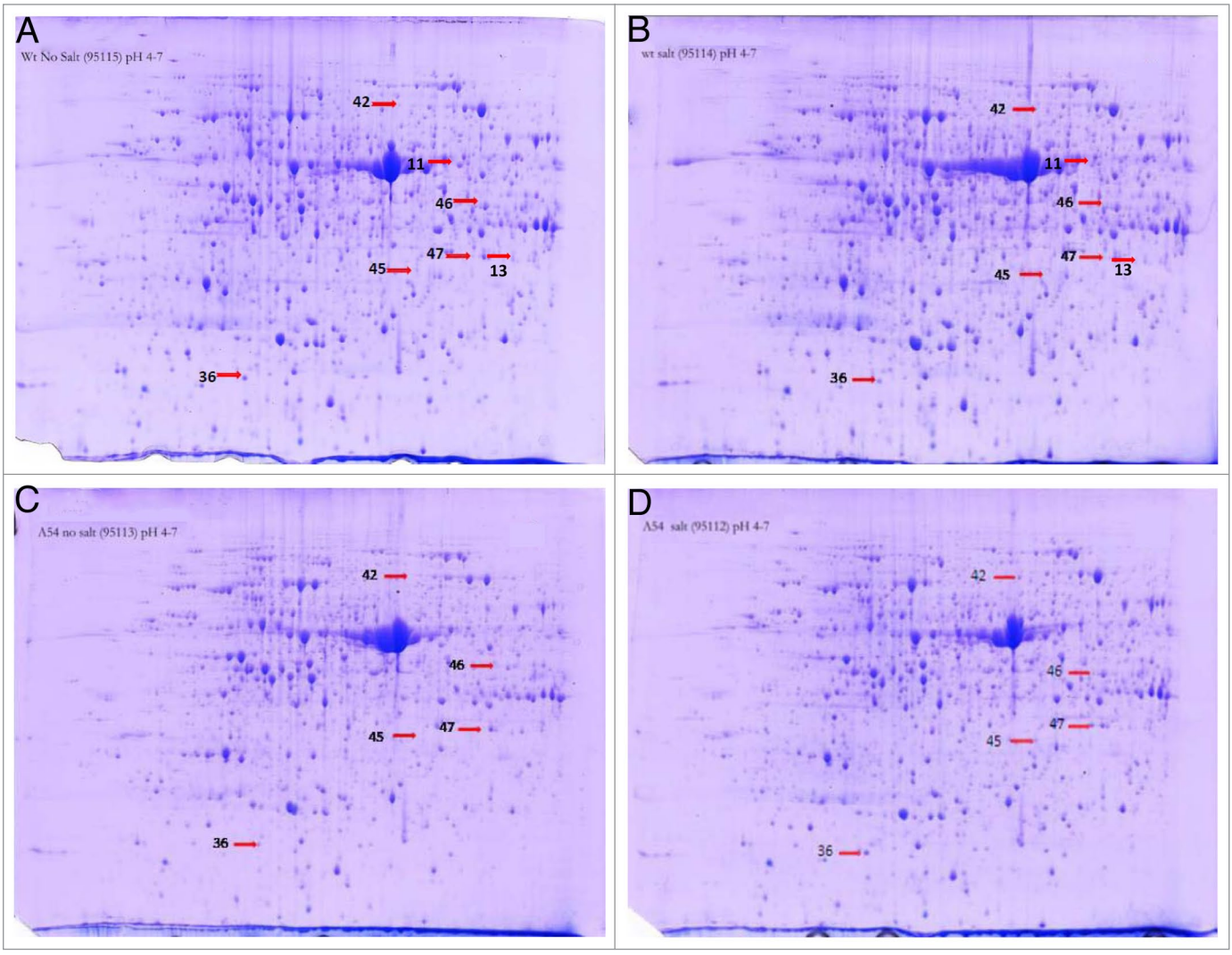

Figure 4. Two dimensional protein gel for wild-type and A54 KO plants treated with 250 mM NaCl for 3 d. (**A**) Wild-type plants without salt treatment. (**B**) Wild-type plants with salt treatment. (**C**) A54 KO plants without salt treatment. (**D**) A54 KO plants with salt treatment. The indicated spots are the spots identified on the gel D that were upregulated relative to gel C. This experiment was performed three times with similar results.

salt, particularly in light of its impact upon drought.[41] It is interesting to note that gene co-expression analysis seems to indicate a negative correlation between AtMKK1 and AtFDH (**Fig. 6B**). It is unclear whether under salt treatment in A54 plants, where AtMKK1 pathway is blocked, these plants activated other pathways, which not only increased the level of FDH (**Fig. 5**) but also led to other changes in these plants, which may contribute to salt tolerance. Gene co-expression analysis has also indicated that a fumarylacetoacetate hydrolase is among the seven proteins likely directly interacting with AtMKK1 (**Fig. 6A** and **Table 2**). Fumarylacetoacetate hydrolase represents the last enzyme of Tyr catabolism, producing the Kreb's cycle metabolite fumarate, which accumulates during seed desiccation.[42]

The impact of salt stress upon the production of energy responsive proteins in the AtMKK1 knockout line is not entirely unexpected. A large number of salt tolerant plants show an elevated expression level of energy related proteins, including proton pumps/ATP synthases as well as proteins involved in the electron transport chain.[43,44] As a common response of plant cells to salt stress, there is a consistent need for higher levels of energy so that the plants can activate transport of an ion against a chemical gradient.[31]

Other proteins responded to salt in wild-type plants. Within the limited number of spots analyzed by mass spectrometry, ATG2 and a S5 ribosomal protein were found downregulated. Although the two genes were not analyzed in depth in our study, previous work may suggest how they could be involved in salt response. ATG2 is an *Arabidopsis* gene specifically involved in autophagy, particularly in roots.[49] Autophagy is a procedure in which a double membrane forms encapsulating some part of the cellular environment for nutrient recycling or to sequester or remove some unneeded cellular components.[50,51] This movement of cellular components creates a variety of diverse roles and activities that autophagy can be involved in ranging from development, nutrient recycling, senescence, as well as a negative regulator of programmed cell death in response to the hypersensitive

Table 1. Identified spots, accession numbers, peptides, protein scores obtained from 2D gels and mass spectrometry

Spot no.	GI	Possible function	Score	Protein coverage	No. of peptides	Up/Down	Peptides	Ions score (Mascot)[1]	E value (Mascot)
36	gi|18491181	Putative H^+ transporting ATP synthase	391	66%	4	Up	LEQ VFS DPQ VLN FFA NPT ITV EK	64	8.8e-4
							QLE DIA SQL ELG EIQ LAT	72	1.3e-4
42	gi|18421656	EMB1467 (embryo defective 1467) NADH dehydrogenase	999	43%	10	Up	ATE TID VSD AVG SNI R	112	1.2e-8
							EGT YEN TEG FTQ QTV PAV PTV GDA R	88	4.2e-6
46	gi|154241492	Formate dehydrogenase	322	28%	4	Up	FVE DLN EML PK	98	3e-7
							CDV IVI NMP LTE K	57	3.9e-4

[1]Mascot Ions score is -10*Log(P), where P is the probability that the observed match is a random event. Individual ions scores > 50 indicate identity or extensive homology ($p < 0.05$).

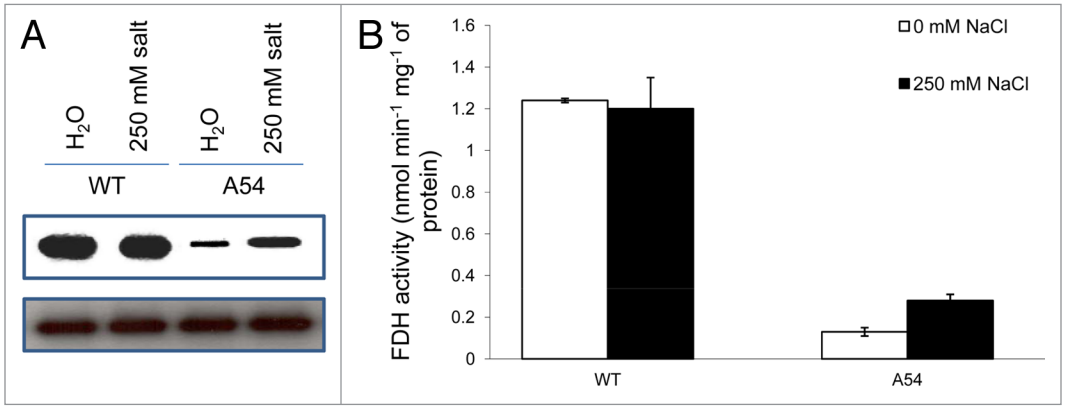

Figure 5. (**A**) Immunological detection of AtFDH after 250 mM NaCl treatment for 3 d of wild-type and A54 KO plants using western blotting and the antibody against AtFDH. The blot indicates the cross reaction of the extracted proteins and anti-AtFDH antibody (upper panel). *Arabidopsis* actin was used as internal protein standard and the blot indicates the cross reaction of the extracted proteins and anti-actin antibody (lower panel). Three experiments were performed with similar results. (**B**) Effect of the same salt treatment as in (**A**) on NAD-dependent FDH enzyme activity. Values are the mean ± standard deviation from three independent experiments (n = 3).

response.[49-53] The decrease of ATG2 protein indicates that there is a possible decrease in the ability of the cell to produce autophagic vesicles. The specific role that ATG2 may play in the response to elevated salt stress remains to be clarified. The pressures of surviving the salt stress may suppress the growth of the vacuole, or require less autophagy in general. Autophagy is often involved in senescence; the altered ability of AtMKK1 knockout mutants to survive in salt may be due to the suppression of ATG2 or other related autophagy genes in an attempt to evade the induction of senescence.

The final identified protein displaying altered expression after salt stress was that of a ribosomal S5 family protein identified as AT2G33800. Ribosomal S5 proteins are important in protein synthesis. S5 proteins play a role in ensuring high translational fidelity during protein synthesis.[54] This is a crucial role; if there is poor fidelity during translation then proper protein function can be decreased or even eliminated. The level of S5 protein in AtMKK1 protein extracts decreased after salt treatment. The level of S5 proteins also decreased after treatment of cabbage leaf curl virus on wild-type *Arabidopsis*.[55] Levels of S5 were also decreased in *mekk1* mutants, an upstream activator of AtMKK1, but not in AtMKK1 mutants indicating that the decrease in S5 levels seen after salt exposure is related to the salt stress and perhaps not a general defense response.[7,12] A major identifiable trait following the induction of salt stress was the diminished growth seen in the salt treated plants. This is an expected outcome as salt suppresses the ability of plants to continue to grow.[31] This could explain the decline in ribosomal S5, if the plants are not growing, the need for ribosomes and subsequent protein synthesis will decline.

In conclusion, AtMKK1 has been reported as a responsive gene in salt stress but it has also been reported as having no role in the salt stress pathway.[3,9,11] The ability of the AtMKK1 mutants to germinate in highly saline environments to a level far above that of the wild-type, as well as the ability of adult plants to resist the effect of high salinity, indicates that AtMKK1 does play a role in salt stress response. AtMKK1 is likely a negative regulator of salt stress, which could indicate why some previous studies have shown it to have no involvement in salt response.[9,10] AtMKK1 is an interesting protein with potentially broad reaching effects on numerous stress response and developmental pathways. The potential avenues to continue studying AtMKK1 are varied and

continuing research into this protein will likely provide a wealth of information that can clear up the somewhat murky MAPK field in *Arabidopsis* and other plant species.

Materials and Methods

Selection of mutant plants and plant growth conditions. T-DNA insertion mutant was obtained from the ARBC (The *Arabidopsis* Resource Centre; www.Arabidopsis.org). AT4G26070 is the locus identification number for AtMKK1 available on the TAIR site (www.Arabidopsis.org). SALK_140054 seeds were obtained from the ABRC. SALK_140054 is found in the third intron.[13]

Mutants were screened on selective media (kanamycin resistance screening). Mutant and wild-type *Arabidopsis* (Col-0) were grown under the same conditions. Following a four-day stratification period, plants were moved to the growth chamber (ENCONAIR Technologies Inc.) and grown under a 16 h light and 8 h darkness cycle at 22°C. After seven days plants that showed kanamycin resistance were transferred to autoclaved soil. One leaf from 3-week-old plants was used for screening by PCR to ensure homozygosity. SALK_140054 plants were re-plated on plates with lacking kanamycin and then planted in the growth chamber and a leaf was collected after three weeks for screening.

Primers for SALK_140054 were created using the SALK Signal iSect primer design tool; the primers obtained were as follows based on their position up- or downstream of the insertion point. The left or upstream primer was 5'-TCC TCT TGA GCA ATC CAT CTC-3' and the right, or downstream primer was 5'-GCC CAC GAA AGA ATT AGC AA-3'. The last primer used to identify knockouts was the Signal LBA1 primer 5'-TGG TTC ACG TAG TGG GCC ATC G-3'. Two sets of PCR were run. One using the left and right primers to identify wild-type alleles and the second using the left primer with the LBA1 primer to identify the T-DNA insertions. The knockout line named A54 was used in all subsequent experiments.

The expression of AtMKK1 in wild-type *Arabidopsis* and in line A54 was examined by PCR according to Gao et al.[45] The forward primer was 5'-AAG CTT ATG AAC AGA GGA AGC TTA TGC CC-3' and the reverse primer was 5'GGA TCC TCA CCA TTG CGA GAT GAA GGA GC-3' for AtMKK1 and 5'-CCT CAT GCC ATC CTC CGT CTTG-3' and 5'-TTC CAT CTC CTG CTC GTA GTC AAC-3' for actin 2 (At3g18780). PCR was performed under the following conditions: 94°C for 3 min; 30 sec at 94°C, 30 sec at 59°C and 30 sec at 72°C for 25 cycles; and then 10 min at 72°C.

Table 2. Genes that are directly related to AtMKK1 (analyzed using Atted-II database)

MR	Cor	Locus	Function
1.7	0.73	At1g74440	Unknown protein
2.0	0.74	At4g08470	MAPKKK10
3.7	0.70	At2g40600	Appr-1-p processing enzyme family protein
4.0	0.66	At4g36150	Disease resistance protein (TIR-NBS-LRR class)
7.2	0.63	At1g11330	S-locus lectin protein kinase family protein
8.4	0.67	At2g37710	RLK (receptor lectin kinase)
16.0	0.45	At3g16700	Fumarylacetoacetate hydrolase family protein

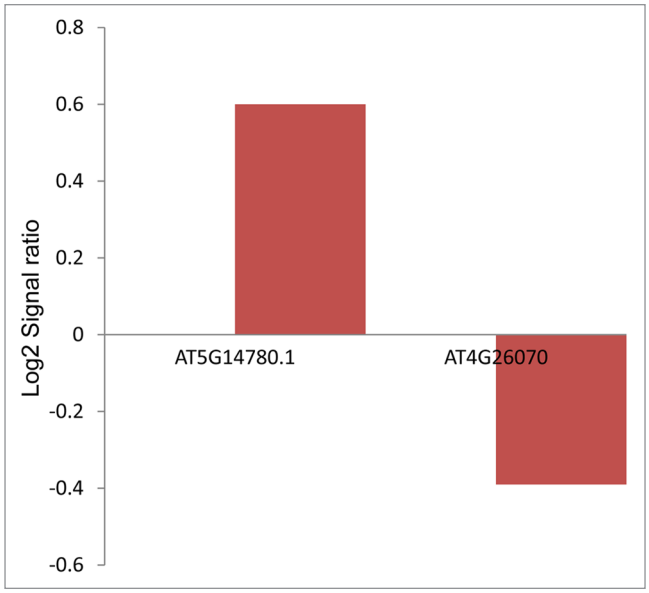

Figure 6. Effect of 24 h treatment with 250 mM NaCl on the expression of AtFDH and AtMKK1 in *Arabidopsis* seedlings. The figure was generated using data obtained from Genevestigator database. Single experiment; no error value provided.

Salt stress treatment. Two separate salinity experiments were performed to assess germination and adult responses to high salinity. For seed germination rates, MS plates were amended with either 125 mM or 250 mM NaCl. A high salinity stress can be obtained by using 250 mM NaCl.[46] An intermediate level was selected to determine if there would be variation due to alteration of the NaCl concentration. After four days at 4°C the seeds were plated on the experimental plates and then placed in the growth chamber. To test salt effect on adult plants, after ten days on the plates the seedlings were transported into autoclaved soil and maintained for four weeks with watering as needed. After four weeks the plants were watered by immersing the trays in NaCl solutions of indicated concentration for the experimental treatment and distilled water for the control plants. This watering procedure was continued every 24 h. This experiment was repeated three times.

Electrolyte leakage analysis. Ion leakage was analyzed according to the method of Shou et al.[47] After leaves were rinsed with distilled H_2O, they were cut into discs (6 mm in diameter) and placed in a glass tube containing 15 mL of distilled water. The test tubes were subjected to vacuum three times at 5 min intervals at 60 psi to remove air bubbles adhered to the surface of the leaves. The tubes were shaken for 2 h at room temperature, followed by the measurement of the conductivity of the solution, which represented the initial conductivity. The solutions were then boiled at 100°C for 30 min to completely disrupt the cell structure. The electrolyte conductivities of the boiled solution were taken as the absolute conductivity after cooling to room temperature. The percentage of electrolyte leakage was calculated by dividing the initial conductivity by the absolute conductivity.

Two-dimensional gel electrophoresis. As salt effects appeared at day 4, day 3 plants were removed from the soil and leaves were flash frozen in liquid nitrogen for protein extraction. Leaves (0.6 g) were ground with a pestle and mortar in liquid nitrogen until a fine powder was formed. Ground tissue was transferred into 50 mL low-binding Falcon tubes. Eight mL of acetone containing 10% (v/v) trichloroacetic acid (TCA) and 0.07% (w/v) dithiothreitol (DTT) was chilled to -20°C and was added to the protein sample while vortexing, the resulting mix was kept at -20°C for two hours. The sample was vortexed every 10 min and centrifuged at 10,000 g for 20 min and the supernatant was carefully removed and discarded. The pellet was washed 8× in acetone with 0.07% (w/v) DTT.

Samples were dried under nitrogen gas and stored at -80°C until needed. Acetone powders were then dissolved in 1 mL IEF solution [(7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 20 mM DTT and 0.5% (v/v) ampholyte, pH 3–10 (Bio-Rad Laboratories)] as described previously[48] and then sonicated for a total of 25 sec with 5 sec pulses using a microtip (Misonix). Samples were then centrifuged at 10,000 g for 20 min and the supernatant was collected and exchanged three times in IEF solution using 5,000 MWC filter devices (Vivaspin, ThermoFisher Scientific). The final volume was brought to 0.4 mL, centrifuged at 80,000 g and the final protein content was determined using a Bradford dye-binding assay.

Samples containing 500/450 μL of protein were used to rehydrate 24 cm IEF strips with pH ranges of 4–7 and 3–10 (GE Healthcare), in a Teflon reswelling tray at 22°C under a layer of mineral oil (DryStrip Cover Fluid, GE Healthcare). Strips were then focused for a total of 58.3 kVh. After focusing, strips were removed, equilibrated as recommended by the manufacturer and placed onto second dimension 12% SDS-PAGE gels. SDS-PAGE was run in standard Tris-glycine buffer using an Ettan Dalt-6 unit (GE Healthcare), at a constant power setting of 0.5 W per gel, 30 min followed by 17 W per gel, until the bromophenol blue dye front had left the gel. Gels were stained using Coomassie brilliant blue R-250 and scanned to produce TIF images, which were printed and analyzed manually. Spots of interest were excised and analyzed by LC-MS. Protein spots were digested in situ with trypsin (Promega) and peptides dissolved in 2% (v/v) acetonitrile (ACN), 1% (v/v) formic acid (FA) and 0.5% (v/v) acetic acid in preparation for LC-MS analysis. Peptides were separated by reversed-phase nano-scale liquid chromatography (LC, Ultimate 3000, Dionex) eluting into linear ion trap mass spectrometer (LTQ: ThermoFisher Scientific). A 10 cm C_{18} column (5 μm particle/300 Å pores) was prepared in-house and used to introduce peptides into the mass spectrometer via nano-spray ionization at 250 nL/min using a 4% to 80% (v/v) ACN gradient in 1% (v/v) FA and 0.5% (v/v) acetic acid over 65 min. The LTQ was programmed to acquire a full survey scan (m/z 300–2,000), to select the 5 most abundant ions from this and perform, serially, an MS^2 scan on each. Former precursor ions were excluded from analysis for 60 sec. Collision energy for MS^2 was 30.0 (isolation width 2.5), tuned with [Glu1]-fibrinopeptide B (m/z = 785). The output files were queried with Mascot (v2.2, Matrixscience) against the NCBI database limited to *Arabidopsis thaliana*. Proteins were considered correctly identified if more than 2 peptides matched with significant Mascot ions scores.

Immunoblotting. A GST-AtFDH polyclonal antibody used was raised in rabbit followed by purification with IgG affinity chromatography (Sigma), as described previously.[45] Detailed protocols for protein extraction and the determination of concentration were followed.[45] *Arabidopsis* actin was also used as internal protein standard and was detected by immunoblotting using its monoclonal antibody (Sigma). After electrophoresis (Mini-PROTEAN 3 System, Bio-Rad Laboratories Inc.), proteins were transferred onto PVDF membranes (Bio-Rad Laboratories) by wet transfer with pre-chilled transfer buffer containing 25 mM Tris (pH 8.5), 192 mM glycine and 20% (v/v) methanol at 4°C for 120 min at 70 V. The primary antibody was used at 1:500 (v/v). After overnight incubation, blots were washed with TBST [(20 mM Tris, pH 7.5, 150 mM NaCl, 0.05% (v/v) Tween-20] (5 min × 3) and then incubated at room temperature for 1 h with 1:2,000 (v/v) dilution of the secondary antibody (for AtFDH using anti-rabbit IgG, horse radish peroxidase-linked and for actin using anti-mouse IgG, horse radish peroxidase-linked, both from Cell Signaling Technology). The target protein on the PVDF membrane was detected using an enhanced chemiluminescence (ECL) system containing 1× LumiGLO Reagent and 1× peroxide (Cell Signaling Technology). The membrane was scanned using FluorChem Q imaging system (Alpha Innotech Cooperation).

Formate dehydrogenase enzyme assay. NAD-dependent formate dehydrogenase (FDH) enzyme activity was determined according to Olson et al.[37] Protein concentration in tissue extracts was determined using a Bradford dye-binding assay.

Bioinformatic analysis. For microarray data mining Genevestigator (www.genevestigator. com) was used. The data were manually recorded and then plotted using Microsoft Office Excel 2007. Protein-protein interactions were predicted using Atted-II 6.0 (http://atted.jp) and STRING 9.0 (http://string-db.org) databases.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Material

Supplemental material may be downloaded here: www.landesbioscience.com/journals/psb/article/24206/

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